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THE GROWTH OF OLFACTORY NEURONS IN SHORT-TERM CULTURES OF RAT OLFACTORY EPITHELIUM

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We have found that purified astrocytes will support the growth of olfactory epithelial neurons (OENs) in vitro. Candidate OENs were visible by light microscopy within one day after plating of dissociated cells from neonatal rat ethmoturbinates. Electron microscopy showed that OENs expressed the unique ultrastructural features which characterize these cells in situ, showing that regulation of neuronal morphology and ultrastructure can function independently of contact with other cells of the appropriate tissue. Characteristics of the cultures are such that this in vitro system would be useful in electrophysiological investigations of the chemosensory olfactory neurons.

The primary chemosensory neurons of the olfactory epithelium are the only population of neurons continuously replaced throughout adult life. Complete cycles of development, beginning with dividing neuronal precursors and continuing through neuronal maturation to cell death, occur over periods of 3–4 weeks [5, 11]. These cycles of loss and replacement mean that denervation and appropriate reinnervation of the olfactory bulb must constantly be recurring [11]. Thus, the olfactory neuroepithelium is a useful model system for studying several major aspects of neuronal development and synaptogenesis. Olfactory neurons are also of particular physiological interest, because the mechanism by which they discriminate different odors is unknown; it is not yet even established whether single neurons respond to single odors or instead recognize a variety of odor stimuli.

A major obstacle to the usefulness of the olfactory epithelium in studies of neurogenesis, or of the electrophysiology of chemosensory transduction, has been the inability to grow olfactory epithelial neurons (OENs) in tissue culture as single cells, free of supporting sustenacular cells, other neurons and the nasal mucosa. As a first step towards investigating the regulation of OEN development, we have developed techniques for growing these cells in vitro. In this paper we describe the growth and ultrastructure of OEN grown on monolayers of purified astrocytes, a culture system in which these neurons can be identified within one day after plating and are subsequently accessible for physiological, morphological and immunochemical studies. In addition, we have found that even when removed from their normal site of growth and complement of supporting cells, OENs express the unique ultrastructural features which characterize these neurons in situ.

Purified cortical astrocytes were prepared by a modification [12] of methods described previously [9]. Purified astrocytes were passaged once in tissue culture flasks, and then grown as monolayers on poly-L-lysine coated glass coverslips. Astrocytes obtained in this way produced flat monolayers, with good visualization of added neurons. Greater than 95% of the cells labeled with rabbit anti-glial fibrillary acidic protein antiserum (a specific marker for astrocytes [1, 13]), and there were no neurofilament$^+$ cells (i.e. neurons [15]), galactocerebroside$^+$ cells (i.e. oligodendrocytes [13, 14]) or normal rabbit serum-binding cells (i.e. macrophages/microglia [13]). The non-astrocytes had fibroblast-like morphologies and often expressed cell-surface fibronectin.

The nasal ethmoturbinates were dissected from newborn Sprague-Dawley rats (N. Klein et al., in preparation) and mechanically dissociated into single cell suspensions [6]. The yield of cells per animal obtained was low ($\approx 2.5 \times 10^5$/rat), but mechanical dissociation offered the major advantage of allowing easy separation of associated cartilage by passage through 45-μm mesh nylon gauze. Cells were diluted to 2, 5 or 10 $\times$ 10^4 cells/ml in Dulbecco's Modified Eagle's Medium (DMEM) containing 5% horse serum, 5% fetal calf serum, 2 mM glutamine and 25 μg/ml gentamicin. Cell suspensions were plated in 0.5 ml aliquots onto astrocyte monolayers growing on glass coverslips in Falcon Multiwell Trays (No. 3047). Cultures were grown for 48–72 h before fixation and preparation for electron microscopy (see Figs. 1 and 2).

Within 24 h after plating, many cells in the cultures possessed morphological features in common with OENs in situ. Candidate OENs were bipolar, with one short process (< 30 μm) terminating in a bulbous expansion and a longer (> 250 μm) and thinner process, each originating from opposite poles of an elliptical (ca. 17 $\times$ 6 μm) cell body. The number of such cells per culture was usually 1–2% of the total number of neuroepithelial cells originally plated. No bipolar cells were found in cultures of bronchial respiratory epithelium, the most likely contaminating tissue in this preparation, indicating that these cells were probably derived from olfactory neuroepithelium. To confirm this, an ultrastructural comparison was performed between candidate OENs and OENs of the newborn rat ethmoturbinate.

Mature OENs in situ display a stereotyped and distinctive ultrastructure [5] (Fig. 1). The major distinguishing feature is the olfactory club (Fig. 1a), which is located at the tip of the apical dendrite and projects into the olfactory mucosa. The club contains numerous centrioles/basal bodies, and often possesses ciliary rootlets and cilia. The dendrite itself is usually 15–30 μm long and 1–3 μm in diameter and contains numerous microtubules and long slender mitochondria aligned along its long axis (Fig. 1b). Centrioles/basal bodies are often seen in the dendrites of less mature neurons, presumably in transit to the sensory club. The dendrites contain no 10-nm neurofilaments. The elliptical cell body is centrally placed in the epithelium and typically measures 17 $\times$ 6 nm (Fig. 1c). The cytoplasm contains abundant free ribosomes, with a sparse polar distribution of other organelles, including rough endoplasmic reticulum (rER) and mitochondria with Golgi apparatus evident proximal to processes in younger cells. The nucleus is ovoid, with large clumps of chromatin

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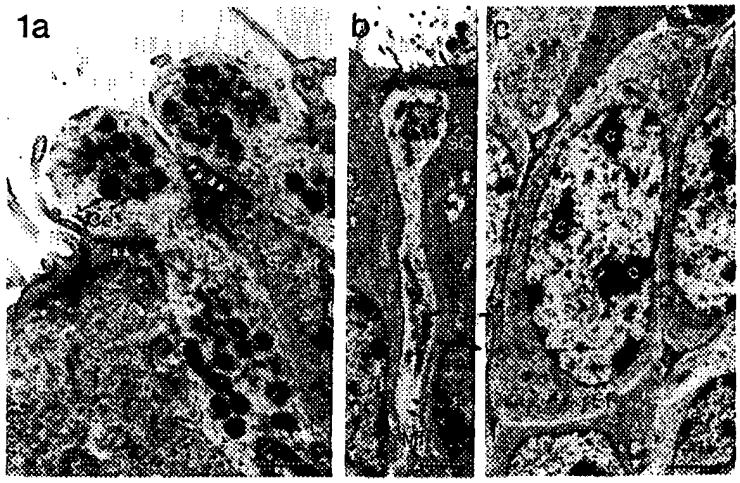


Fig. 1. The figure shows the fine structure of olfactory neurons in situ. Tissue was processed for electron microscopy by immersion prefixation in 2.5% glutaraldehyde for 2 h followed by post-fixation in 2.0% OsO_4, also for 2 h. Both fixatives were buffered in PBS (Dulbecco 'A'). After dehydration in ethanol, specimens were embedded in Araldite. a: two sensory clubs are shown projecting from the free surface of the epithelium. Abundant centrioles are seen (Ce) both in, and en route to, the club. Both clubs make extensive contact (white triangles) with supporting cells (S Cell). Bar = 0.5 μm. b: a low power micrograph showing a dendrite whose sensory club (SC) has not yet penetrated the free surface, as confirmed by serial section analysis (not shown). Elongate mitochondria (M) and microtubules (Mt) are orientated along the dendrite's long axis. Bar = 1.5 μm. c: detail of an olfactory cell body orientated as in (b) and located proximal to the dendrite. Note the single nucleus (N) and the characteristically distributed chromatin (C) located in the nucleus. Sparse organelles, especially rough endoplasmic reticulum (rER) and mitochondria (M), are seen in a polar cytoplasm, with profuse free ribosomes distributed throughout. Bar = 2 μm.

and a single nucleolus distributed in an electronlucent nucleoplasm. A slender axon (0.3–0.5 μm) projects through the basement membrane and lamina propria of the epithelium and eventually to the olfactory bulb.

In vitro, candidate cells expressed the characteristic ultrastructural features of OENs in situ (Fig. 2). A typical pear-shaped bipolar cell is shown in Fig. 2a, with one long slender process (which extends > 250 μm) and a thicker short ($\approx$ 25 μm) process ending in a bulbous expansion; Fig. 2b is an electron microscopic montage of the same cell. The 25-μm process contains numerous aligned microtubules, elongated mitochondria and at least one centriole (Fig. 2c). The most important feature is the club-shaped specialization terminating the 25-μm process (Fig. 2d). This club contains less than 25 centrioles and at least one ciliary rootlet; segregation of centrioles to such a specialization is a unique feature of OENs. The elliptical cell body contains rER profiles and mitochondria, especially proximal to the long process, and profuse free ribosomes. The nucleus possesses the chromatin pattern characteristic of OENs in situ.

Twelve of 15 candidate OENs chosen at the light microscopic level had the defining ultrastructural features of OENs. Two of the negative cells had some, but not

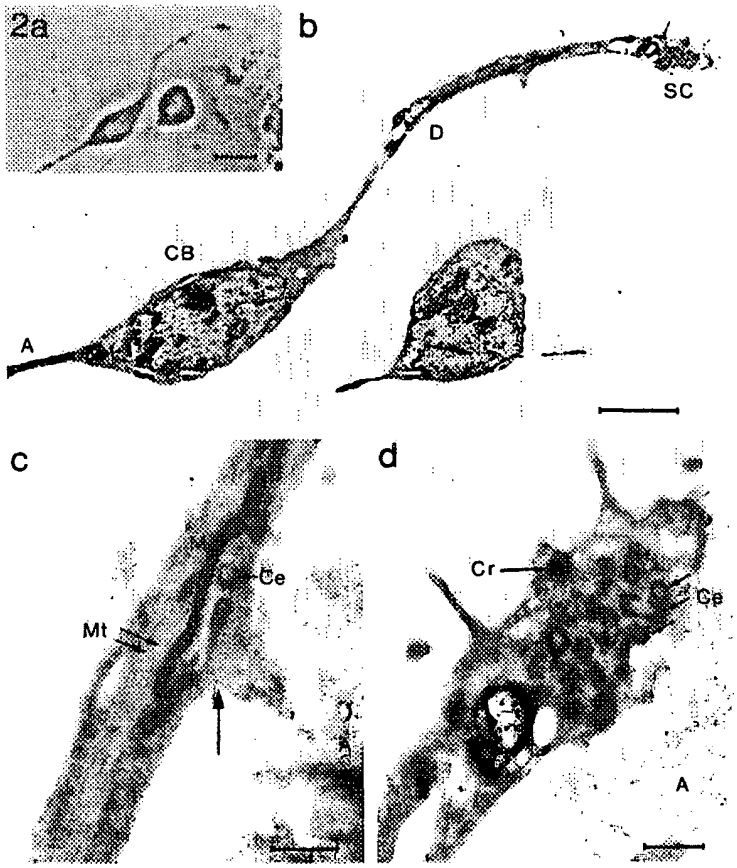
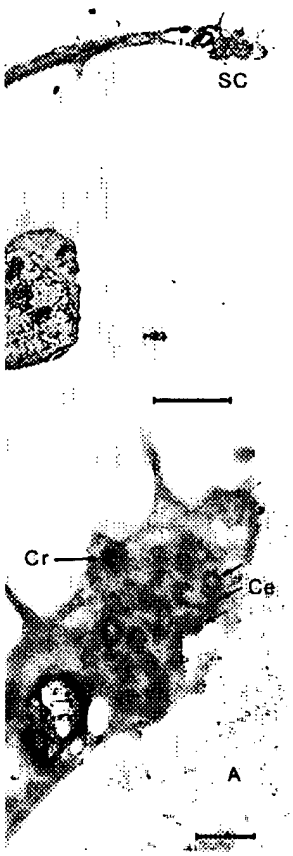


Fig. 2. The figure shows the morphology and fine structure of olfactory neurons in vitro. Cultures were processed for electron microscopy as above, except that the times of each fixation were reduced to 15 min. Cultures and coverslips were embedded in a thin layer of Araldite to facilitate light microscopic study. a: a light micrograph of a candidate OEN lying on an astrocyte. This was photographed in Araldite, then serially sectioned en face for electron microscopy, the coverslip having been removed with hydrofluoric acid. Bar = 10 μm. b: a through-section photomontage of the candidate OEN shown in (a). Note the characteristic elliptical cell body (CB) and nucleus, the dendrite (D) projecting from one pole, and the segregated centrioles in a terminal sensory club (SC). A long, slender axon (A) projects from the opposite pole. The smaller cell may be an immature neuron. Bar = 5 μm. c: detail of the dendrite shows that abundant microtubules (Mt) are present together with slender, elongate mitochondria (M) and a solitary centriole (Ce). A point of contact with the smaller cell is shown (large arrow), as is the astrocyte on which the OEN rests (A). Bar = 0.5 μm. d: detail of the sensory club. The accumulation of centrioles (Ce) is obvious; also present is a ciliary rootlet (Cr). The two filipodial processes and the large inclusion body are culture artefacts. Bar = 0.5 μm.

all of the features of OENs, and the remaining one cell was indistinguishable from a fibroblast. Various minor features poorly seen in the cell in Fig. 2, and associated



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with differing stages of OEN development in situ, were often found in candidate OENs (Fig. 3), suggesting that cultures contained OENs at various stages of maturation. In addition, cells which did not wholly conform to our light microscopic criteria for candidate OENs often displayed various ultrastructural features of OENs. Thus, it is likely that we are underestimating the overall frequency of OENs in our cultures.

The ability to support neuronal growth in vitro is one of the distinguishing characteristics of the astrocyte, and astrocytes have been found to promote the growth of various PNS and CNS neurons [8, 12]. We have now found that astrocytes will support the in vitro growth of OENs, a population of neurons which has proven difficult to grow in tissue culture conditions.

Of particular interest was the observation that OENs could be readily identified by their expression in vitro of ultrastructural features unique to this cell type. It has been observed that some classes of neurons will develop morphologies in vitro similar to those seen in situ [7]. Our findings appear to offer the first demonstrations that the regulation of morphological development can extend to the level of ultrastructure and that this regulation will function independently of contact with other cells of the appropriate tissue.

Will normal cycles of OEN development occur in vitro, with associated cell division, neuronal maturation and neuronal death? One previous study, in which antiserum against olfactory marker protein (OMP) was used to indentify OENs, suggested that in vitro development did not occur [4]. However, it may be that, as with Schwann cells and myelin-specific compounds [2, 10], OMP production requires induction by specific signals, while generation of normal morphology does not. More recent studies [3, 6], in which cultures of olfactory epithelial tissue were followed

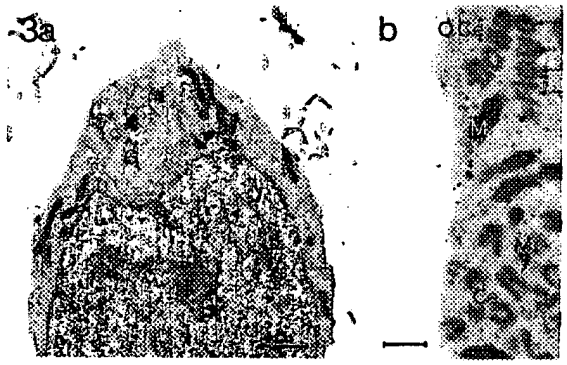

Fig. 3. This figure shows other features of olfactory neurons in vitro. a: an OEN cell body, orientated as in Fig. 1c, showing an extensive Golgi system (G) proximal to the dendrite and club (not shown). Such Golgi systems are often seen in association with growing processes, and its absence in Fig. 1c and 2b suggests that the latter are mature neurons. Bar = 1.5 μm. b: a high power micrograph of part of a sensory club showing elongated mitochondria (M) and microtubules (Mt), with many centrioles (Ce). A group of 8 orientated centrioles (OCe) demonstrate a regularity often seen in situ.

for up to 2 months, suggest that neurogenesis may well occur in vitro under appropriate conditions; our preliminary work on long-term cultures also suggests that neurogenesis occurs in the system reported here. Advantages to growth on astrocyte monolayers are that many OEN cultures can be obtained from 2-3 newborn rats, and OENs grow as single cells easily recognizable by light microscopy within a short time after plating. Thus, this culture system will be useful in selecting antibodies which recognize antigens on the OEN cell surface, and is eminently suitable for electrophysiological studies of odor sensitivities and of chemosensory transduction.

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